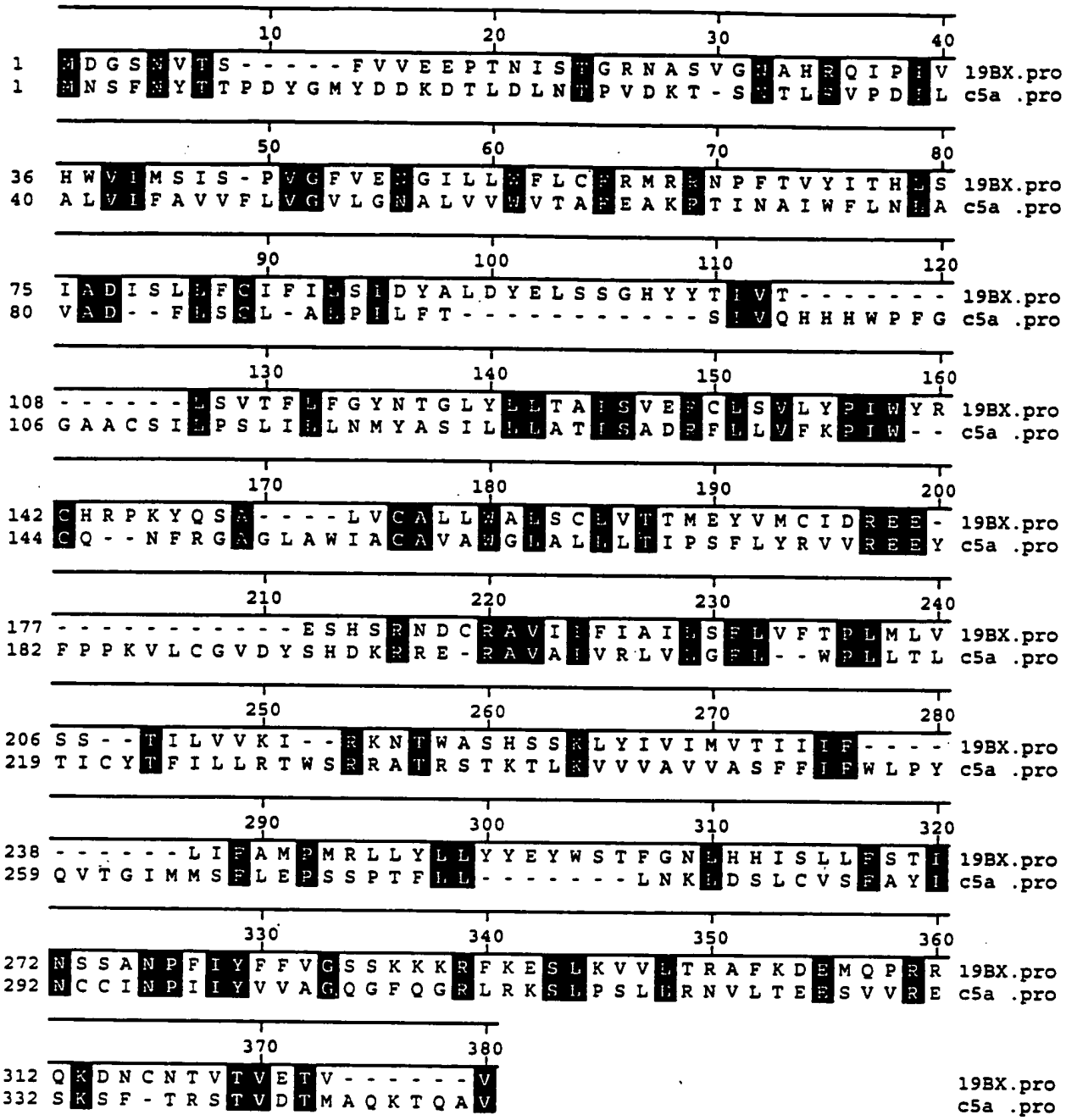
Appendix E

(E1-E8)

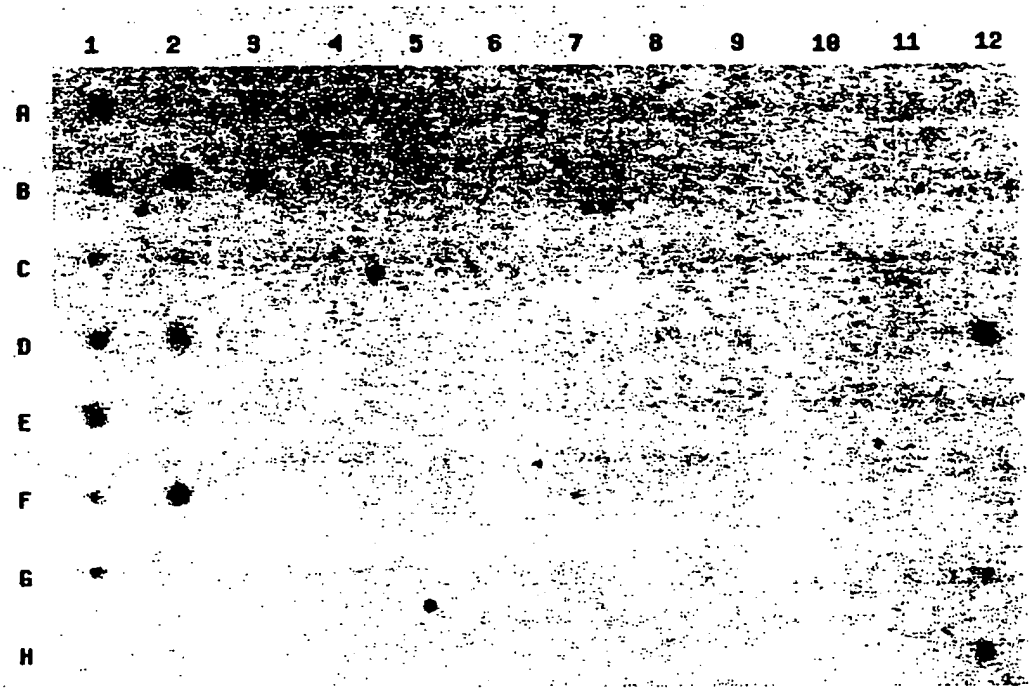
Appendix E1



Decoration 'Decoration #1': Shade (with solid bright cobalt) residues that match the consensus named 'Consensus #1' exactly.

Appendix E

E2



E3

	1	2	3	4	5	6	7	8	9	10	11	12
A		Cerebellum Left	Substantia Nigra	Heart	Esophagus	Colon Transverse	Kidney	Lung	Liver	Leukemia HL-60	Fetal Brain	
B	Cerebral Cortex	Cerebellum Right	Accumbens	Aorta	Stomach	Colon Desending	Skeletal Muscle	Placenta	Pancreas	HeLa S3	Fetal Heart	
C	Frontal Cortex	Corpus Callosum	Thalamus	Atrium Left	Duodenum	Rectum	Spleen	Bladder	Adrenal Gland	Leukemia K562	Fetal Kidney	
D	Parietal Lobe	Amygdala	Pituitary Gland	Atrium Right	Jejunum		Thymus	Uterus	Thyroid	Leukemia MOLT-4	Fetal Liver	
E	Occipital Cortex	Claudete Nucleus	Spinal Cord	Ventricle Left	Ileum		Peripheral Leukocyte	Prostate	Salivary Gland	Burkitt's Lymphoma Raji	Fetal Spleen	
F	Temporal Cortex	Hippocampus		Ventricle Right	Ilocecum		Lymph Node	Testis	Mammary Gland	Burkitt's Lymphoma Daudi	Fetal Thymus	
G	Paracentral Gyrus of Cerebral Cortex	Medulla Oblongata		Inter Ventricular Septum	Appendix		Bone Marrow	Ovary		Colorectal Adenocarcinoma SW480	Fetal Lung	
H	Pons	Putamen		Apex of the Heart	Colon Ascending		Trachea			Lung Carcinoma A549		

Appendix E4

MCA Occlusion/Reperfusion

The rat middle cerebral artery occlusion/reperfusion model was substantially performed as published by Menzies et al (Neurosurgery 1992 Jul;31(1):100-6). Briefly, animals were anaesthetized and a craniotomy of approximately 2X2mm was made in the right squamosal bone. The middle cerebral artery (MCA) was exposed and ligated with a suture for 1hr. After occlusion the suture was removed and reperfusion through the MCA was allowed to occur for various amounts of time (0hrs to 192 hours) after which the animals were sacrificed and the brains harvested. The brains were frozen in isopentane and stored at -86°C until used for cryostat sectioning. Cryostat sections (15um) were mounted on glass slides and then stored at -86°C until used for in situ hybridization studies.

In situ Hybridization Protocol

1. ***Preparation of In Situ Probes***

The in situ probe DNA fragment of rat 19BX was obtained by PCR using the following oligonucleotides:

5'- ACTTTCATGCTTGGTGACCACCATGG-3' (5' oligo)

5'-CCACAGTCTCAATGGATACAGTGTTGCC-3' (3' oligo).

The PCR condition utilized was as follows: the reaction condition utilized was 1X rTth DNA polymerase buffer II, 1.5 mM $Mg(OAc)_2$, 0.2 mM each of the 4 nucleotides, 0.228 μg rat genomic DNA, 0.25 μM of each primer (*see* below) and 1 unit of rTth DNA polymerase (Perkin Elmer) in 50 μl reaction volume. The cycle condition was 30 cycles of 94°C for 1 min, 55 °C for 1 min and 72 °C for 45 sec with a Perkin Elmer Cetus 2400 thermal cycler.

The resulting PCR fragment was then isolated and cloned into the pCRII-TOPO vector (Invitrogen).

2. ***Tissue preparation***

Dissected tissue was frozen in isopentane cooled to -42°C and subsequently stored at -80 °C prior to sectioning on a cryostat maintained at -20°C. Slide-mounted tissue sections were then stored at -80 °C.

3. ***In Situ Hybridization Protocol***

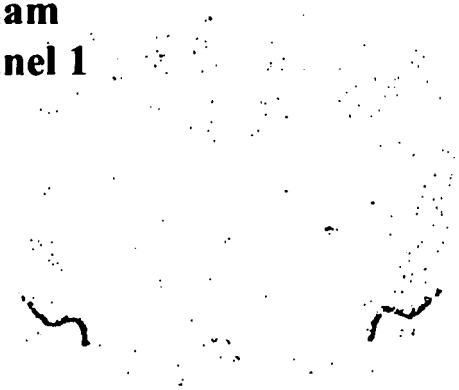
Tissue sections were removed from the -80°C freezer and incubated with a 1 µg/ml solution of proteinase-K to permeabilize the tissue and inactivate endogenous RNase. After this treatment, sections were incubated in succession in water (1 min), 0.1 M triethanolamine (pH 8.0; 1 min), and 0.25% acetic anhydride in 0.1 M triethanolamine (10 min). The tissue was then washed in 2 x SSC (0.3 mM NaCl, 0.03 nM Na citrate, pH 7.2; 5 min) and dehydrated through graded concentrations of ethanol. Sections were then hybridized with 1.5 x 10^6 dpm of [^{35}S]UTP-labeled cRNA probes in 20 µl of a hybridization buffer containing 75% formamide, 10% dextran sulfate, 3 x SSC, 50 mM sodium phosphate buffer (pH 7.4), 1 x Denhart's solution, 0.1 mg/ml yeast tRNA, and 0.1 mg/ml sheared salmon sperm DNA. Tissue sections were covered with coverslips that were sealed with rubber cement. The slides were incubated overnight at 50°C. On the following day, the rubber cement was removed, the coverslips were soaked-off with 2 x SSC, and the tissue sections were washed for 10 min in fresh 2 x SSC solution. Single stranded probe not hybridized with endogenous mRNAs was removed by incubating the sections for 30 min in 200 µg/ml solution of RNase-A at 37°C. The tissue was then washed in increasingly stringent SSC solutions (2, 1 and 0.5 x SSC; 10 min each), followed by a 1 hr wash in 0.5 x SSC at 60°C. After this final wash, the tissue sections were dehydrated using graded concentrations of ethanol, air dried and prepared for detection by x-ray autoradiography on Kodak XAR-5 film.

4. ***Analysis***

Utilizing the above protocol on normal male rats (Sprague-Dawley, Charles River), it was determined that 19BX is expressed in the following areas of the brain: ipsilateral cingulated cortex, in a time dependent manner. *See* panels 1-9 of **Appendix E5**.

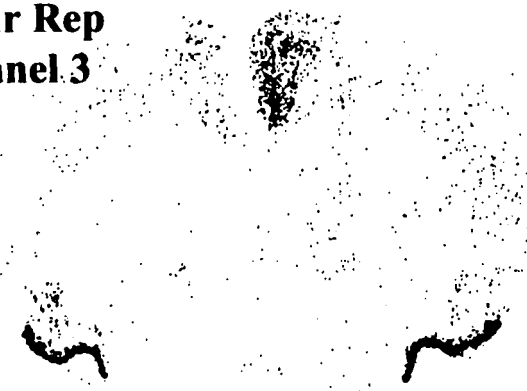
Appendix E5

Sham
Panel 1

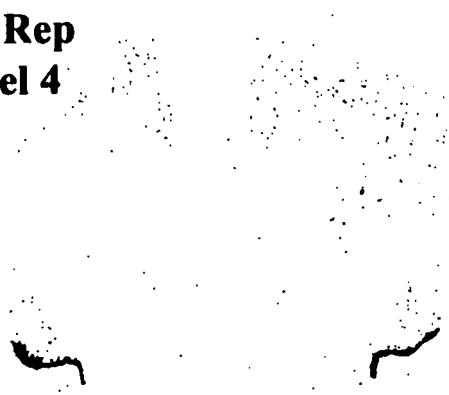


0hr Rep
Panel 2

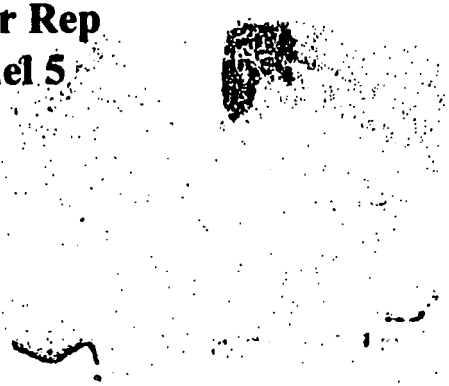
3hr Rep
Panel 3



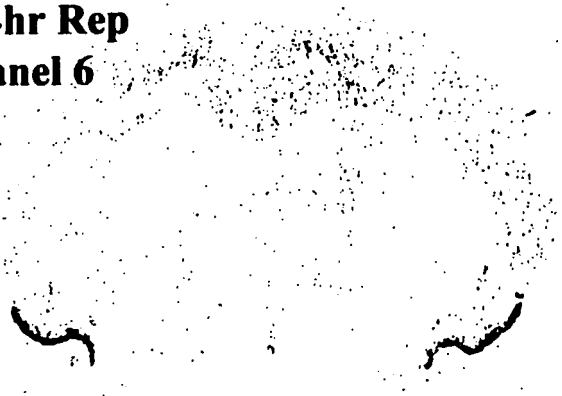
6hr Rep
Panel 4



12hr Rep
Panel 5



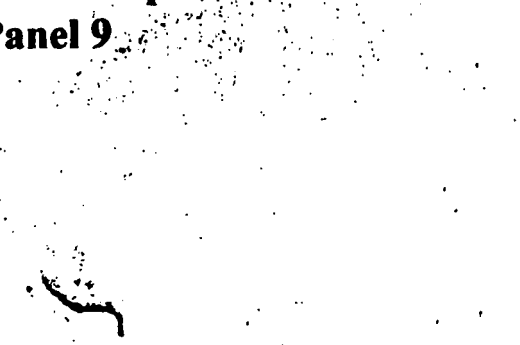
24hr Rep
Panel 6



48hr Rep
Panel 7

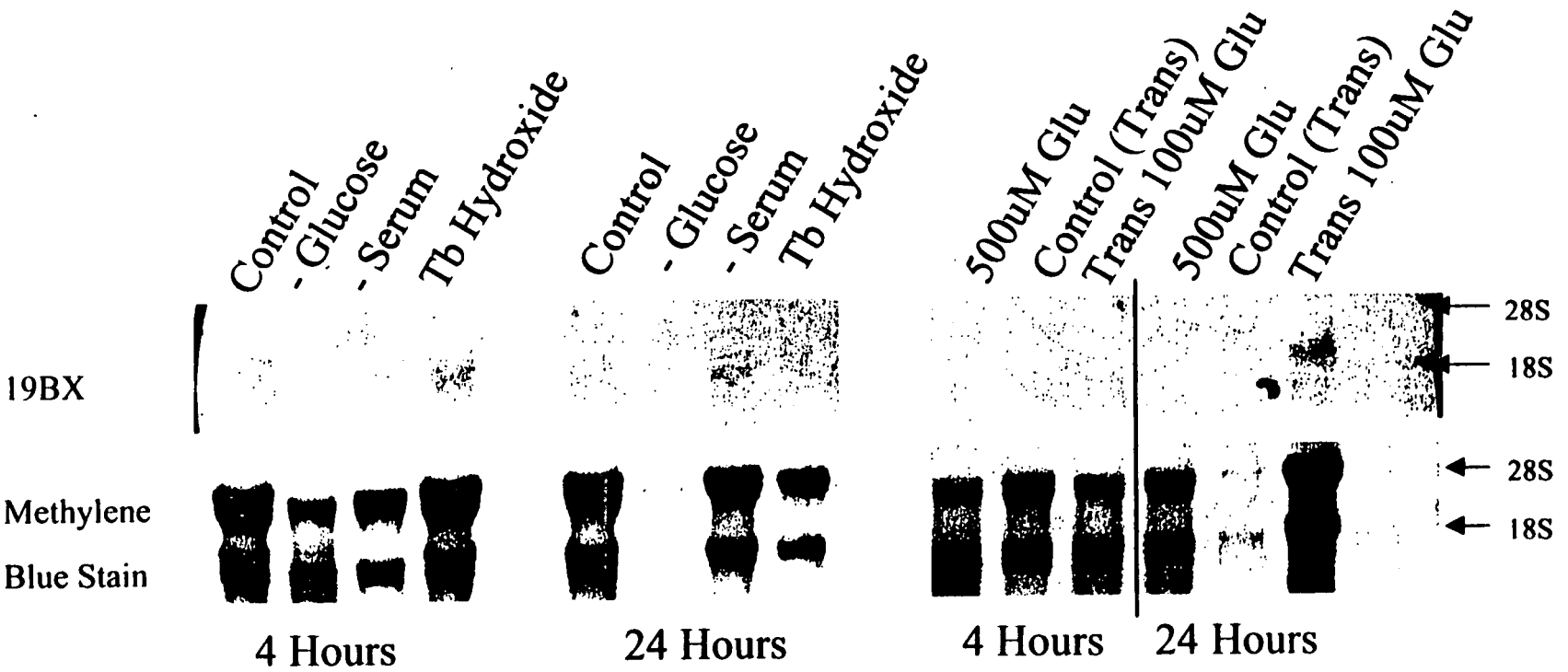
96hr Rep
Panel 8

192hr Rep
Panel 9



Appendix E6

19BX is expressed by primary rat neurons



Appendix E7

AP1 Reporter Assay

A recognized method to detect Gq stimulation depends on the known property of Gq-dependent phospholipase C to cause the activation of genes containing AP1 elements in their promoter. A Pathdetect™ AP-1 cis-Reporting System (Stratagene, Catalogue # 219073) was utilized to assay for Gq coupled activity in 293 cells. Cells were transfected with the plasmid components of the system and the indicated expression plasmid encoding endogenous or non-endogenous receptor using a Mammalian Transfection Kit (Stratagene, Catalogue #200285) according to the manufacturer's instructions. Briefly, 410 ng pAP1-Luc, 80 ng CMV-receptor expression plasmid and 20 ng CMV-SEAP (secreted alkaline phosphatase expression plasmid; alkaline phosphatase activity is measured in the media of transfected cells to control for variations in transfection efficiency between samples) were combined in a calcium phosphate precipitate as per the manufacturer's instructions. Half of the precipitate was equally distributed over 3 wells in a 96-well plate, kept on the cells overnight, and replaced with fresh medium the following day. 48 hr after the start of the transfection, cells were treated and assayed for luciferase activity using a Luclite™ Kit (Packard, Cat. # 6016911) and "Trilux 1450 Microbeta" liquid scintillation and luminescence counter (Wallac) as per the manufacturer's instructions. The data was analyzed using GraphPad Prism™ 2.0a (GraphPad Software Inc.).

Appendix E8

Activation of AP1/Luciferase Reporter Gene Expression in 293 Cells Indicates 19BX Signals Primarily Via Gq